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(54) Title: FLUORESCENCE POLARIZATION–BASED DIAGNOSTIC ASSAY FOR *LEPTOSPIRA* SEROVARS

(57) Abstract

In a homogeneous immunoassay for leptospirosis, antibodies specific for antigens present on the FlaB protein of *Leptospira* serovars bind to a diagnostic reagent comprising a recombinant FlaB protein labeled with fluorescein isothiocyanate, to give a detectable increase in fluorescence polarization. The assay is fast, simple to perform, and safe.

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FLUORESCENCE POLARIZATION-BASED DIAGNOSTIC ASSAY FOR LEPTOSPIRA SEROVARS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/101,788, filed on September 25, 1998.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a homogeneous immunoassay for detection of antibodies to a range of leptospira serovars. A fluorescent antigen probe is provided as a diagnostic reagent that, when combined with the target antibodies, shows an increase in fluorescence polarization, thereby quantitating the antibody present.

2. Description of Related Art

Leptospirosis is a zoonotic infection, which exhibits a broad spectrum of clinical manifestations, ranging in severity from acute to chronic with multi-organ syndrome, to fatal. Leptospirosis affects wild rodents and domestic animals such as cattle, swine, horses, sheep, goats and dogs. Infections with leptospires also represent an occupational hazard to farmers, butchers and veterinarians. The pathogenic leptospires were formerly classified as *Leptospira interrogans*. but the genus has recently been reorganized and pathogenic leptospires are now identified in seven species of *Leptospira*. These bacteria are divided in to 23 serogroups and subdivided into approximately 212 serovars on the basis of common cross-reacting agglutinins. For a review see Faine, S., *Leptospira and Leptospirosis*, CRC Press Inc., Boca Raton, Florida (1994).

The diagnosis of leptospirosis depends either on the detection of antibodies in the sera or the presence of the organisms in tissues or body fluids. Since the isolation of

leptospires is difficult and laborious, serological diagnosis is extensively used. The microscopic agglutination test (MAT) is the most commonly used diagnostic test, as described in Cole, et al., *Appl. Microbiol.*, 25:976 (1973). Several other techniques, such as the passive hemagglutination test, the immunofluorescence test, and enzyme immunoassays, have also been investigated. However, these assays have significant drawbacks, such as the use of a battery of live leptospires, with an associated risk of a laboratory-acquired infection; involvement of multiple reagents and steps. There is a need to provide a diagnostic laboratory test which can detect anti-*Leptospira* antibodies in biological fluids with high sensitivity and specificity, and which can be performed within a short period of time. Although the enzyme-linked assay (ELISA) meets most of the desired requirements, improved simplicity and rapidity are still needed.

Dandliker et al., introduced a homogeneous fluorescence polarization assay (FPA) that is sensitive, specific, rapid and requires few reagents (Dandliker, et al., *Immunochem,* 10:219 (1973)). Fluorescence polarization is a measure of the time-averaged rotational motion of fluorescent molecules. When a fluorescently-labeled antigen binds to the antibody, its fluorescence polarization will increase due to the larger hydrodynamic volume of the antigen-antibody complex. The FPA has been applied to the quantitation of analytes, investigation of protein-protein interaction, ligand binding, enzymatic activity, and monitoring of the interaction between antibody and its epitope.

Nielsen et al., *J. Immunol. Methods.* 195:161 (1996), reported the development of a FPA using fluorescein-labeled Brucella abortus O-polysaccharide as a tracer antigen, to detect Brucella antibodies in bovine sera. The authors demonstrated that FPA was a highly accurate assay with a sensitivity and specificity of 99.02% and 99.96%, respectively. In a similar study, Lin et al., *Clin. Diag. Lab. Immunol.*, 4:438 (1996)

reported the use of fluorescein-labeled *Mycobacterium bovis* secretory protein MPB70 as a tracer-antigen in a FPA. The authors indicated that the FPA was able to detect anti-MPB70 antibodies in the sera of infected animals but not in the sera of uninfected animals.

Information on the nature of leptospiral antigens is important to elucidate their significance in the immunity, pathogenesis and diagnosis of leptospirosis. Leptospires have a characteristic corkscrew-like movement, which is mediated by two *periplasmic flagella* (PF) inserted subterminally into the protoplasmic cylinder. Sequence analysis of flagellar genes and proteins from several spirochetes show that there are two distinct classes of proteins, FlaA and FlaB in the filament. FlaA proteins are associated with the sheath surrounding a core that is composed of FlaB proteins. Electrophoresis has revealed that the PF is composed of three prominent proteins with molecular weights of 31, 37 and a 33 - 34 kDa doublet (See Kelson, *J. Med. Microbiol.*, 26:47 (1987)). Additionally, several proteins with molecular weights between 30-67 kDa have been identified from *L. interrogans* serovar *hardjo* (See Nunes-Edwards, et al., *Infect. Immun.*, 48:492-497 (1985)). However, the significance of these proteins in diagnosis, pathogenesis or immunity to infection is not yet clear.

Recently, a 35 kDa protein from *L. interrogans* serovar *pomona* that is recognized by mono- and polyclonal anti-*Leptospria* antibodies has been identified. This protein was identified as the flagellin protein FlaB in *L. interrogans* serovar *pomona*, and the *flaB* gene encoding this protein was also identified. This work is described in Lin, et al., *Infect. Immun.*, 65:4355-4359 (1997), which is incorporated herein by reference.

However, despite the recent elucidation of various *Leptospira* serovar proteins, a need exists to provide a diagnostic assay that can detect anti-*Leptospira* antibodies in

biological fluids with high sensitivity and specificity, so that infected animals can be identified quickly, reliably, and easily.

SUMMARY OF THE INVENTION

In a first principal aspect, the invention provides a fluorescent antigen probe comprising a fluorophore conjugated to a purified and isolated protein comprising a sequence of amino acids that is at least 98.6% homologous with the sequence of amino acids set forth in SEQ ID NO:2. The fluorescent antigen probe binds to serum antibodies to *Leptospira* serovars to produce a detectable change in fluorescence polarization.

In a second principal aspect, the invention provides an assay for serum antibodies reactive with an antigen common to a range of *Leptospira* serovars. A serum specimen suspected of containing antibodies reactive with an antigen of *Leptospira* is diluted with a buffer solution, to provide a buffered specimen. A fluorescent antigen probe is added to this buffered specimen. The fluorescent antigen probe comprises a fluorophore conjugated to a purified and isolated protein comprising a sequence of amino acids that is at least 98.6% homologous with the sequence of amino acids set forth in SEQ ID NO:2. The buffered specimen with added probe is incubated for a time sufficient to permit binding in solution of the antibodies to the antigen probe to provide a reaction product. The fluorescence polarization of this reaction product is compared to a blank control.

In a third principal aspect, the invention provides a diagnostic assay kit for detecting serum antibodies to a range of *Leptospira* serovars. The kit includes a fluorescent antigen probe in an amount suitable for at least one assay and suitable packaging. The fluorescent antigen probe comprises a fluorophore conjugated to a purified and isolated protein comprising a sequence of amino acids that is at least

98.6% homologous with the sequence of amino acids set forth in SEQ ID NO:2.

In accordance with preferred embodiments of the present invention, the fluorescence polarization-based diagnostic assay, utilizing a fluorescent antigen probe, is rapid, easy to use, and has a high sensitivity to and specificity for a range of *Leptospira* serovars.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph showing the results of SDS-PAGE applied to samples from five steps in the purification process of the FlaB protein used to make the fluorescent antigen probe in accordance with a preferred embodiment of the present invention.

Figure 2A is a photograph of the gel under UV illumination showing the results of SDS-PAGE analysis of labeled and unlabeled recombinant FlaB protein.

Figure 2B is a photograph of the gel of Figure 2B after Coomassie blue staining.

Figure 3A is a photograph showing the results of a Western blot analysis of both labeled FlaB and unlabeled FlaB probed with bovine sera containing antibodies against serovar *pomona*.

Figure 3B is a photograph showing the results of a Western blot analysis of both labeled FlaB and unlabeled FlaB probed with bovine sera containing antibodies against serovar *hardjo*.

Figure 3C is a photograph showing the results of a Western blot analysis of both labeled FlaB and unlabeled FlaB probed with bovine sera containing antibodies against serovar *sejroe*.

Figure 4 is a graph showing the receiver operating characteristic (ROC) curve for

the fluorescence polarization assay, in accordance with a preferred embodiment of the present invention, applied to 208 MAT-positive and 208 MAT-negative bovine serum samples.

Figure 5 is a bar graph showing the results of the fluorescence polarization assay, in accordance with a preferred embodiment of the present invention, as applied to 208 MAT-positive and 208 MAT-negative bovine serum samples.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

1. Identification of the leptospiral 35-kDa protein.

L. interrogans was cultured at 29°C in SPL 5X *Leptospira* medium (Scientific Protein Laboratories, Waunakee, Wisconsin). Whole-cell antigens were prepared from *L. interrogans* serovar *pomona* and analyzed by Western blotting with M138 monoclonal antibody (2 μg/ml) as the probe. The results showed immunoreactive bands of various molecular sizes, including a strong immunoreactive band of approximately 35-kDa, which also reacted strongly with the antisera from naturally *L. interrogans* infected cattle. Protease K treatment of the serovar *pomona* antigens eliminated the immunoreaction between M138 and the antigens, suggesting that M138 recognized a protein epitope in nature. Although the reason for the reaction of M138 with many leptospiral antigens on Western blots was unclear, the 35-kDa protein that reacted strongly with M138 was targeted for further characterization.

The 35-kDa protein was highly purified from an outer sheath antigen preparation (see Auran et al., *Infect. Immun.* 5:968 (1972)) through a high-performance liquid chromatography size exclusion column (TSK G2000 SWG; 21.5 by 600 mm; Phenomenex, Torrance, Calif.) with 50 mM ammonium acetate as the elution buffer. The

purified serovar *pomona* protein antigens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto a polyvinylidene difluoride membrane, and excised for the determination of the N-terminal amino acid sequence. Amino acid sequencing was performed on a model 470A gas phase sequencer equipped with an on-line model 120A PTH analyzer (Applied Biosystems, Foster City, Calif.). The N-terminal amino acid sequence found for this protein is provided herein as SEQ ID NO:1.

A similarity search of the N-terminal amino acid sequence of the 35-kDa protein was performed against the sequence databases through the National Center for Biotechnology Information (NCBI) BLAST E-mail server. A striking homology (61.5 to 92.2% identity) between the N-terminal amino acid sequence (13 amino acids) of the serovar *pomona* protein and those of the flagellins from other bacteria was found. This considerably high level of homology suggested that the 35-kDa protein is a constituent polypeptide of PF. The PF from several different spirochetes has been found to be complex structures consisting of several different polypeptide subunits. The PF filaments consist of a core structure surrounded either by one outer sheath layer as in *Spirochaeta aurantia* and *Treponema pallidum* or by two outer layers as in *L. interrogans*. Sequence analyses of the PF genes and proteins from several spirochetal sources have shown that there are two distinct classes of proteins, namely FlaA and FlaB, which form the outer sheath and core layers of the PF filament, respectively. The 35-kDa protein described here is a FlaB protein based upon its N-terminal amino acid sequence, and its gene is therefore referred to as *flaB*.

2. Cloning and sequence analysis of the serovar *pomona* flagellin gene.

The total genomic DNA was extracted from the cell cultures with a Rapidprep Macro Genomic DNA isolation kit (Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). To analyze the gene encoding the leptospiral flagellin that reacted strongly with M138, PCR was employed to amplify the gene homolog from the serovar *pomona* genomic DNA with a pair of oligonucleotide primers: 5'GTGGAGCTCATGATTATCAA(T/C)CA(C/T)AA(C/T)CT3' and 5'ACAGGATCCTCAGAT(A/G)TGCTGCAGAAG(C/T)TT3'. The PCR primers, which contain the *Sac*I and *Bam*HI cognition sequences at their 5' ends, were derived, respectively, from the N-terminal amino acid sequence of the serovar *pomona* 35-kDa protein and the conserved C-terminal amino acid sequence of a leptospiral periplasmic flagellar subunit (see Mitchison, et al., *J. Gen. Microbiol.*, 137:1529 (1991)). PCR was performed with *Taq* DNA polymerase (Life Technologies, Burlington, Ontario, Canada). Following a hot start (85°C for 5 minutes) and denaturation at 94°C for 30 seconds, 40 cycles of amplification at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute were performed. The PCR resulted in a single DNA fragment of approximately 850 bp. which presumably corresponds to the open reading frame of the flagellin. With the same pair of primers, an 850-bp fragment was also amplified by PCR from the genomic DNA of serovars *hardjo*, *autumnalis*, and *copenhageni*. DNA sequence analysis revealed that these PCR products were the flagellin gene homologs. These results suggest that the FlaB protein is highly conserved among pathogenic serovars of *Leptospira*. The highly conserved FlaB in leptospires may explain the observation that M138 cross-reacted with various pathogenic serovars, and the cross-reactivity of periplasmic flagellar proteins among various strains of *Leptospira* has been previously described.

The *Sac*I and *Bam*HI-digested 850-bp amplified gene fragment from the serovar *pomona* genomic DNA was ligated into pUC 118 at *Sac*I and *Bam*HI sites and cloned in *Escherichia coli* TG1. Recombinant plasmids pUC118pomFla-1 and pUC118pomFla-2, prepared from the cultures of two selected white colonies, were restriction analyzed with *Sac*I and *Bam*HI. Both recombinant plasmids contain an insert with an apparent molecular size corresponding to that of the 850-bp amplified gene. DNA sequencing was performed on an ABI model 373 automatic sequencer with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Foster City, Calif.) according to the manufacturer's instructions. The recombinant plasmid DNA was used as sequencing templates. Both strands of the DNA insert were initially sequenced with pUC/M13 forward and reverse primers, 5'GTAAAACGACGGCCAGT3' and 5'CAGGAAACAGCTATGAC3', respectively, and completed with an additional pair of internal primers, 5'TTATAATAAGCTCCCATATC3' and 5'TACTGAAGACGGAATGAGTT3', synthesized according to the initial nucleotide sequence obtained with the pUC/M13 primers.

The inserted DNA fragment contains an open reading frame of 849 bp with a G + C content of 46.88% which codes for a 283-amino-acid (aa) protein. The encoded protein contains 31 (10.95%) acidic (D and E), 34 (12.0%) basic (K and R) , and 102 (36.0%) hydrophobic (A, I, L, F, W, and V) amino acids. The deduced protein sequence contains no cysteine residue and only one tryptophan residue. The calculated molecular mass and predicted pI value of the encoded protein were 31.297 kDa and 9.065, respectively. The G + C content of the serovar *pomona flaB* gene is significantly lower than the 54.7% found for serovar *hardjo* FlaB but higher than the 39% G + C content of the leptospiral genome reported in LeFebvre, et al., *J. Clin. Microbiol.* 25:2094-2097 (1987). The

deduced protein sequence is provided herein as SEQ ID NO:2. The nucleotide sequence for the *flaB* gene is provided herein as SEQ ID NO:3.

The calculated molecular mass of the serovar *pomona* FlaB is remarkably similar to that reported for the serovar *hardjo* FlaB (see Mitchison, et al., *J. Gen. Microbiol.*, 137:1529-1530 (1991)), but it is smaller than the size of the native protein (35 kDa) as determined by SDS-PAGE. At least two possibilities may account for this difference. Molecular mass estimation with SDS-PAGE may not be precisely accurate. Alternatively, there may be posttranslational modification of the serovar *pomona* FlaB protein in vivo. Such posttranslational modifications are found with the FlaB polypeptides of other spirochetes, as described in Charon, et al., *Res. Microbiol.*, 143:597-603 (1992). In particular, the addition of a carbohydrate group to the serovar *pomona* flagellin is possible.

Nucleotide and protein sequences were analyzed and aligned with a Lasergene software package (DNASTAR, Inc., Madison, Wisconsin). The serovar *pomona* flagellin amino acid sequence, deduced from the nucleotide sequence, was aligned with the sequences of flagellins from several spirochetes and with *Bacillus* sp. strain C-125. The serovar *pomona* flagellin shows a close relationship with the serovar *hardjo* protein, with a homology (identity) of 98.6%. In comparison with other bacterial species, the serovar *pomona* protein has an overall 42.0% identity with the *Bacillus* sp. strain C-125 flagellin, 39.6% identity with the flagellins from two *Borrelia* species (*B. hermsii* and *B. burgdorferi*), and 55.5% to 57.2% identity with the *T. pallidum* flagellins (FlaB1, FlaB2, and FlaB3).

3. Production of Recombinant FlaB Protein.

PCR amplification of pUC118pomFla-1 gave a product of 870 bp, comprising the *flaB* structural gene and a restriction endonuclease recognition sequence at each end to allow cloning into the vector, pProEx HT. The pProEx HT expression vector was obtained from Life Technologies (Burlington, Ontario, Canada). This plasmid contains the *lacI*^{q} gene, thereby enabling expression, induced by isopropyl-β-D-thiogalactoside (IPTG), of the inserted gene from the *trc* promoter. By inserting the *flaB* structural gene downstream in frame with the initiation codon of a coding sequence for MSYYHHHHHHDYDIPTTENLYFQGAMGS, wherein the underlined portions are a six-histidine tag and a Tobacco Etch Virus (TEV) protease cleavage site. respectively, an expression vector (designated pHTFlaB) was constructed. The pHTFlaB construct enables production of a FlaB fusion protein with a polyhistidine tag. The polyhistidine tag facilitates affinity purification of the desired protein and may be removed by proteolysis with TEV protease.

The expression construct pHTFlaB, generated as previously described, was utilized to produce the recombinant FlaB in *E. coli* DH5α. Briefly, Luria-Bertani (LB) broth (500 ml) supplemented with ampicillin (100 μg/ml) was inoculated with 5 ml of an overnight culture of the *E. coli* harboring pHTFlaB. Cultures were grown at 37°C in an incubator (Environ Shaker) until an optical density at 590 nm of 0.8-1.0 (approximate time 4 to 5 hours) was achieved. Expression of FlaB was initiated by the addition of IPTG at a final concentration of 0.6 mM. Cultures were incubated for an additional 3 hours to allow maximum protein expression. The cells were harvested by centrifugation at 10,000 x *g* for 20 minutes at 4°C. The cell pellet was stored at -80°C until further analysis. In order to confirm protein expression, a 1.0 ml aliquot of the *E. coli* culture was taken before and after adding IPTG and was analyzed by electrophoresis.

4. Purification of recombinant FlaB protein.

The recombinant FlaB protein was purified by affinity chromatography on a nickel-nitriloacetic acid (Ni-NTA)-agarose column followed by electroelution. Samples from each step in the purification process of recombinant FlaB protein were subjected to SDS-PAGE, and the results are shown in Figure 1. Lane 1 shows the results for soluble lysate of uninduced *E. coli*; lane 2 the results for soluble lysate of induced *E. coli*; lane 3 the results for the crude extract of induced cells prior to Ni-NTA affinity chromatography; lane 4 the results for the proteins eluted from the Ni-NTA column with 80 mM imidazole; and lane 5 the results for purified FlaB following electroelution. The positions of the molecular mass markers are indicated in Figure 1, and the recombinant FlaB protein is indicated by an arrowhead. Lanes 1 and 2 contain total cell proteins from 1 ml of culture with an A_{590} of 0.2.

The SDS-PAGE was performed by the method of Laemmli in 12% (wt/vol) resolving and 4% (wt/vol) stacking polyacrylamide gels, using a mini-PROTEAN II gel apparatus (Bio-Rad). After electrophoresis, the proteins were either stained with Coomassie brilliant blue or electrophoretically transblotted onto nitrocellulose membrane (NC) (Bio-Rad) using a Trans-Blot Cell (Hoeffer Scientific) at 100 volts for 2.5 hours with cooling. The NC was blocked overnight with Tris-buffered saline supplemented with Tween 20 (10 mM Tris-HCl, 140 mM NaCl, pH 7.4, 0.5% Tween) (TBS-T) plus 5% heat-inactivated horse serum (Armour, Kankakee, Illinois). After blocking, the NC was reacted with diluted bovine antiserum (1:100) for 3 hours at room temperature and then with anti-bovine immunoglobulin (IgG) conjugated with a horseradish peroxidase (1:5000). The NC was washed (five times) between each reaction step with TBS-T.

Bound conjugate was visualized with a horseradish peroxidase substrate kit (Bio-Rad). The molecular weights of the separated proteins were determined by comparison with prestained protein standards (Bio-Rad).

The first step in the purification process is obtaining the lysate from *E. coli*. Briefly, IPTG-induced *E. coli* cells harboring pHTFlaB were resuspended in 10 ml of buffer A (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris-HCl, pH 8.0), lysed for one hour at room temperature with continuous stirring, and then kept overnight at 4°C. Insoluble material was removed by centrifugation at 27,000 x *g* for 40 minutes at 4°C to obtain the supernatant.

SDS-PAGE analysis of the total lysate of *E. coli* harboring pHTFlaB demonstrated that the recombinant FlaB protein showed up as a strong band at the 31 kDa position in the cells induced with IPTG (Figure 1, lane 2). The 31 kDa position is expected for this protein based on the molecular weight calculated for its deduced amino acid sequence. However, FlaB was not detected in the *E. coli* extracts grown in the absence of IPTG (Figure 1, lane 1). It was also observed that the FlaB was contaminated with several high and low molecular weight proteins of *E. coli*, as shown in lanes 2 and 3 of Figure 1.

The recombinant protein FlaB was partially purified by Ni-NTA affinity chromatography (See Hochuli, E., p. 87-98, in Setlow, J.K. (ed.), *Genetic engineering, principle and method*, Plenum, New York (1990); and Holzinger, et al., *News Qiagen*, 4:14-15 (1996)) according to the manufacturer's instructions (Qiagen). The supernatant was applied to an Ni-NTA column (1 x 7.5 cm) pre-equilibrated with buffer A. The *E. coli* proteins were eluted by sequential washing with progressively acidic buffers, namely, 50 ml each of buffer B (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris-HCl, 1 M NaCl, pH 6.3),

buffer C (same composition as buffer B except pH 5.9). Selective elution of FlaB protein was achieved with buffer D (same composition as buffer C except containing 80 mM imidazole). The fractions were collected and the absorbance was measured at 280 nm in a spectrophotometer (Pharmacia LKB Ultrospec plus). All eluted fractions were subjected to SDS-PAGE to confirm the presence of FlaB protein. The peak fractions containing the FlaB protein were pooled and stored at -20°C.

The elution profile at 280 nm showed 2 well-separated peaks. SDS-PAGE analysis demonstrated the presence of *E. coli* proteins in the fractions of the first peak and the presence of a strong band in the 31 kDA region (corresponding to the molecular weight of FlaB) in the second peak. Affinity purified FlaB was found to be contaminated with one higher and one lower molecular weight band (Figure 1, lane 4).

The FlaB was further purified by electroelution. Following Ni-NTA affinity chromatography, the partially purified FlaB preparation was subjected to SDS-PAGE as described above. Individual protein bands were visualized by staining with Tris-Coomassie buffer (0.125 M Tris-HCl, pH 6.8) containing 0.2% Coomassie brilliant blue, and destaining with 0.125 M Tris-HCl (pH 6.8) containing 0.1% SDS and 1 mM EDTA. The FlaB protein band was excised and electroeluted with a Bio-Rad Electroelutor model 422 at a constant current of 10 mA/tube for 4 to 6 hours at room temperature using elution buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.5). The purified FlaB was concentrated in an Ultrafiltration Stirred Cell (Amicon) fitted with a PM-10 (25 mm) ultrafiltration membrane (Amicon). Purified FlaB was dialyzed overnight against 0.02 M phosphate-buffered saline (PBS), pH 7.2, containing 0.01% SDS.

In general, the elution time for maximal recovery of FlaB was 6 hours. A purified product represented by a strong single band at the 31-kDa position was obtained, with no

detectable contamination bands (Figure 1, lane 5). Purified FlaB was then concentrated, dialyzed, and labeled with fluorescein dye.

5. FITC labeling.

Purified FlaB (650 μg in 1 ml) mixed with 0.3 ml of a 0.15 M sodium phosphate buffer (pH 9.5), containing fluorescein isothiocyanate (FITC) in a concentration of 1 mg/ml, was used for the FITC labeling. The reaction mixture was incubated for 3 hours at 37°C, though incubation periods of 2 to 7 hours at temperatures of 25°C to 41°C could also be used.

In order to separate free dye from the labeled FlaB protein, the reaction mixture was applied to a Sephadex G-25 column (1 x 23 cm)(Pharmacia), pre-equilibrated with 0.1 M sodium phosphate buffer (pH 7.0). The absorbance of the fractions was monitored at 492 nm (Pharmacia LKB Ultrospec plus). Eluted fractions were pooled and analyzed by SDS-PAGE. The protein bands were visualized by UV light (Transilluminator, VWR Scientific) followed by staining with Coomassie brilliant blue.

Analysis of the Sephadex G-25 gel filtration chromatography fractions at 492 nm revealed the presence of two distinct peaks. The fractions of the first peak contained a bluish-green fluorescent eluate. The second peak contained free FITC.

The covalent attachment of FITC to FlaB was confirmed by SDS-PAGE, as shown in Figures 2A and 2B. Figure 2A is a photograph of the gel taken under UV illumination, and Figure 2B shows the same gel after Coomassie blue staining. In Figures 2A and 2B, lane 1 shows the results for the reaction mixture comprising FlaB and FITC; lane 2 shows the results for unlabeled FlaB; and lane 3 shows the results after gel filtration chromatography. An arrowhead indicates the recombinant FlaB protein in

Figures 2A and 2B. The gels exposed to UV light prior to staining revealed the presence of a fluorescently labeled doublet at the 31 kDa site (See Figure 2A, lanes 1 and 3), corresponding to the position of unlabeled FlaB (Figure 2B, lane 2). Unlabeled FlaB migrated as a single band of an apparent molecular weight of 31 kDa (Figure 2B, lane 2). The fractions of the first peak containing FITC-labeled FlaB were pooled and used as the tracer-antigen, as described below in more detail.

The antigenicity of labeled and unlabeled FlaB was confirmed by Western blotting using Bovine sera containing antibodies against *L. interrogans* serovar *pomona,* and *L. borgpetersenii* serovars *hardjo* and *sejroe.* Sera from uninfected animals were used as negative controls. Western blot analysis revealed that both labeled and unlabeled FlaB were recognized by anti-Leptospira antibodies against various serovars, thus indicating the broad antigenic cross-reactivity of this recombinant protein and indicating that the labeling did not effect the antigenicity. Figures 3A, 3B, and 3C show the Western blot results, wherein unlabeled FlaB (lane 1) and labeled FlaB (lane 2) were electrophoresed on 12% SDS-polyacrylamide gel, transblotted onto NC and probed with bovine sera containing containing antibodies against serovar *pomona* (Figure 3A), serovar *hardjo* (Figure 3B), and serovar *sejroe* (Figure 3C). In Figures 3A-3C, the recombinant protein FlaB is indicated by an arrowhead.

In the case of unlabeled FlaB, a serum sample with a high titer of antibodies against *pomona*, as determined by MAT, recognized a single band at the 31-kDa site (Figure 3A, lane 1). However, in the case of labeled FlaB, the same serum sample reacted with a doublet of the 31-kDa position (FIG. 3A, lane 2), with the same intensity. Similar patterns were obtained with serum samples with high titers of antibodies against serovars *hardjo* and *sejroe* (Figures 3B and C, respectively). This suggests that the

purified unlabeled and labeled FlaB were immunologically active, and the immunogenicity of the epitope was not altered by modification with fluorescein. However, the reason for the appearance of the doublet in the case of labeled FlaB is unclear. It is possible that the labeling resulted in at least two populations of labeled proteins with different degrees of substitution. The MAT-negative serum samples did not react with the labeled or unlabeled FlaB.

6. Fluorescence Polarization Assay (FPA).

The FPA was performed using FITC-labeled FlaB as a tracer antigen. The FPA was carried out on a FPM-1 Fluorescence Polarization Analyzer (Jolley Consulting and Research, Inc., Grayslake, Illinois). The baseline for the tracer antigen alone was established with 0.1 M PBS (pH 7.2). Serum samples were diluted (1:50) in 0.01 M PBS containing 0.1% sodium azide and 0.05% lithium dodecyl sulfate. Two milliliters of diluent were dispensed into a glass tube (12 x 75 cm). Test serum (0.04 ml) was then added to the diluent tube and mixed thoroughly. Each sample was blanked in the fluorescence polarization analyzer. The predetermined amount of the tracer antigen (0.02 ml) was then added to the serum sample and mixed thoroughly. The mixture was allowed to equilibrate for 10 to 30 minutes at room temperature. Following the equilibration period, the fluorescence polarization was measured as millipolarization units (mP) in a fluorescence polarization analyzer.

A mean fluorescence millipolarization (mP) value of 147 (n=4) was obtained when the fluorescence polarization of the tracer antigen diluted in PBS was measured. Initially, five MAT-positive and four MAT-negative bovine serum samples (1:50) were analyzed by FPA. The MAT-positive sera, in the presence of the tracer-antigen, gave a

mean millipolarization value ranging from 201 to 214 (n=5). The MAT-negative sera, in the presence of the tracer-antigen, gave a mean millipolarization value between 151 to 155 (n=4), which was very close to the mP value obtained with the tracer-antigen alone. The same serum samples, when tested by Western blotting with the tracer antigen, confirmed the presence or absence of anti-leptospiral antibodies. It was demonstrated that Western blot data was in agreement with FPA results. These results suggested the feasibility of FITC-labeled recombinant FlaB as a tracer-antigen for the detection of anti-leptospiral antibodies using FPA.

Note that the MAT procedure used herein was a modification of the method previous described in Cole, et al., "Improved microtechnique for the leptospiral microscopic agglutination test," *Appl. Microbiol.*, 25:976-980 (1973). Live 4-day-old *Leptospira* cultures (*Leptospira* serovars *sejroe*, *hardjo*, *pomona*, *canicola*, *icterohaemorrhagiae*, and *grippotyphosa*) were used as antigens. The endpoint titer was the highest dilution of serum that agglutinated at least 50% of the leptospires. Agglutination at a serum dilution of 1:100 was considered positive.

A total of 416 field serum samples were then tested by FPA. All of the serum samples were supplied by Animal Diseases Research Institute, Lethbridge, Canada. All of the sera were initially tested once using the MAT. Because these serum samples had been stored at -20°C for two to three years, they were equilibrated at room temperature before testing in FPA.

A total of 208 sera which were MAT-positive for at least one of the serovars *pomona, sejroe, canicola, icterohaemorrhagiae, grippotyphosa or hardjo*, were used to estimate the relative sensitivity of the FPA, and 208 sera which were MAT-negative for the serovars listed above were used to estimate the relative specificity. The relative

sensitivity and relative specificity were calculated at different cutoff points using receiver operating characteristic (ROC) analysis to evaluate the performance of the FPA for the detection of anti-leptospiral antibodies.

The ROC curve plots the relative sensitivity (true-positive ratio) versus relative specificity (false-positive ratio) while the cut-off value for a positive or negative result is varied, and is completely independent of disease prevalence. The area under the ROC curve provides a quantitative assessment of a test's diagnostic performance, in this case, the ability of FPA to discriminate the sera with or without antibodies. The larger the area under the ROC curve the better the test discriminates. A perfect test has an area of 1.0, whereas a non-informative test has an area of 0.5 or less. The 95% confidence intervals are calculated as indicators of the precision of the relative sensitivity and relative specificity estimates. ROC analysis analyzes the diagnostic performance for the full range of cut-off points, and thus eliminates the bias resulting from selection of a single value.

In this study, a large area (0.90; 95% confidence interval = 0.856 to 0.918) was observed under the ROC curve for FPA (Figure 4), suggesting that the FPA could be a useful test for the serodiagnosis of leptospirosis. It was demonstrated that at low cut-off points, the FPA has a high relative sensitivity and high negative predictive value; however, the relative specificity and positive predictive values were low. At higher cut-off points, although the relative specificity and positive predictive values were high, the relative sensitivity and negative predictive values were low.

The optimum cut-off point for the FPA from the ROC curve was 161 mP, which yielded a relative sensitivity value of 83.7% ± 0.01% and a relative specificity value of 81.2% ± 0.01% (Figure 4 and Table 1). At the 161 mp cut-off, the positive predictive

value was 81.7% and the negative predictive value was 83.8% (Table 1).

The distribution of data obtained with the 416 sera tested with the fluorescence polarization assay described herein is shown in Figure 5. In Figure 5, the sample numbers (y axis) were plotted against the mP value (x axis). The shaded bars show the results for the 208 MAT-positive sera and the unshaded bars show the results for the 208 MAT-negative sera.

Table 1

ROC cut-off (mP)	Relative Sensitivity (%)	Relative Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
121	99.5	15.9	54.2	97.0
141	97.1	40.4	62.0	93.3
151	93.3	66.3	73.5	90.8
161*	83.7	81.2	81.7	83.8
171	62.0	93.3	90.2	71.1
182	28.4	97.1	90.8	57.5
192	7.7	99.0	88.9	51.8

* Cut-off point giving the most efficient screening characteristics of FPA.

The analysis of this FPA data reveals that the present assay provides a valuable addition to the existing diagnostic methods in leptospirosis. The assay is safe since it does not require use of live organisms as antigens. It is sensitive and rapid, typically providing a screening result within 10 minutes. In addition, the tracer antigen and antibodies have an indefinite shelf life with proper storage measures. Finally, the FPA is semi-automated and the data analysis is easy. These qualities indicate that FPA is useful technique in the diagnosis and epidemiological studies of leptospirosis.

Further details regarding this fluorescence polarization-based assay are provided in Bughio, et al., *Clinical and Diagnostic Laboratory Immunology*, 6:599-605 (1999), which is fully incorporated herein by reference.

7. Assay Kit

The fluorescent antigen probe of the present invention is preferably made available in kit form. The kit preferably includes a quantity of buffer solution for diluting serum specimens suspected of containing antibodies to *Leptospira*, the fluorescent antigen probe in amount suitable for at least one assay, along with suitable packaging and instructions for use. The fluorescent antigen probe may be provided in solution, as a liquid dispersion, or as a substantially dry powder (e.g., in lyophilized form).

The suitable packaging can be any solid matrix or material, such as glass, plastic, paper, foil, and the like, capable of separately holding within fixed limits the buffer and the synthetic fluorescent antigen probe. For example, the buffer solution and the fluorescent antigen probe may be provided in separate labeled bottles or vials made of glass or plastic.

The fluorescent antigen probe comprises a fluorophore conjugated to a purified and isolated protein that preferably corresponds to the FlaB flagellar protein of *Leptospira interrogans* serovar *pomona* described herein. This protein may be prepared recombinantly, using the nucleotide sequence set forth in SEQ ID NO:3, and purified using Ni-NTA affinity chromatography, followed by electroelution, as described herein. The deduced amino acid sequence for this flagellar FlaB protein is set forth in SEQ ID NO:2. However, this amino acid sequence was found to be 98.6% homologous to the flagellin protein of another serovar, *hardjo*, suggesting that other proteins that are at least 98.6% homologous with the amino acid sequence set forth in SEQ ID NO:2 could also be used in the fluorescent antigen probe.

A fluorophore is conjugated, i.e., covalently attached, to this FlaB flagellar protein in the fluorescent antigen probe. The fluorophore is preferably fluorescein isothiocyanate. However, other fluorophores, such as rhodamine, BODIPY™, Texas Red™ and Lucifer yellow, could also be used. For a detailed listing of a variety of commercially available fluorophores, see Handbook of Fluorescent Probes and Research Chemicals, ed. Karen Larison, by Richard P. Haughland, Ph.D., 5th ed., 1992, published by Molecular Probes, Inc. The fluorescent antigen probe is preferably prepared by mixing about 0.3 ml of FITC (1 mg/ml) in a 0.15 M sodium phosphate butter (pH 9.5) with about 650 μg of the purified FlaB protein in about 1 ml of buffer, or by a scaled-up version of this procedure. The kit preferably provides the fluorescent antigen probe prepared in this way in an amount suitable for at least one assay, in suitable packaging.

The buffer solution provided in the kit is preferably 0.01 M PBS that also contains 0.1% sodium azide and 0.05% lithium dodecyl sulfate. However, other

buffers could also be used.

The diagnostic assay kit is intended to be used in the following way, as should be described in the instructions for use. A serum specimen suspected of containing antibodies to *Leptospira* is diluted with a quantity of the buffer solution provided in the kit to provide a buffered specimen. A dilution of about 1:50 in a total volume of about 2.0 ml is preferred. The buffered specimen is then blanked in the reader, after which about 0.02 ml of the fluorescent antigen probe is added, the fluorescent antigen probe having been prepared as described above. The buffered specimen with added probe is then incubated for a time sufficient to permit binding in solution of *Leptospira* antibodies with the antigen probe to provide a reaction product. An incubation time of about 10 to 30 minutes at room temperature is typically sufficient. The fluorescence polarization of the reaction product is then compared to a negative serum control, i.e., compared to a buffered solution of serum obtained from an animal known to be free of *Leptospira*, to which the fluorescent antigen probe is added at about the same concentration.

CLAIMS

What is claimed is:

1. A fluorescent antigen probe comprising:

a purified and isolated protein comprising a sequence of amino acids that is at least 98.6% homologous with the sequence of amino acids set forth in SEQ ID NO:2; and

a fluorophore conjugated to said protein, wherein said fluorescent antigen probe binds to serum antibodies to *Leptospira* serovars to produce a detectable change in fluorescence polarization.

2. The fluorescent antigen probe of claim 1, wherein said protein comprises a sequence of amino acids as set forth in SEQ ID NO:1.

3. The fluorescent antigen probe of claim 1, wherein said protein comprises a sequence of amino acids as set forth in SEQ ID NO:2.

4. The fluorescent antigen probe of claim 1, wherein said fluorophore is fluorescein isothiocyanate.

5. An assay for serum antibodies reactive with an antigen common to a range of *Leptospira* serovars comprising the steps of:

diluting a serum specimen suspected of containing antibodies reactive with an antigen of *Leptospira* with a buffer solution, to provide a buffered specimen;

adding to said buffered specimen a fluorescent antigen probe comprising a fluorophore conjugated to a purified and isolated protein comprising a sequence of

amino acids that is at least 98.6% homologous with the sequence of amino acids set forth in SEQ ID NO:2;

incubating for a time sufficient to permit binding in solution of said antibodies to said antigen probe to provide a reaction product; and

comparing the fluorescence polarization of said reaction product to a control.

6. The assay of claim 5, wherein said protein comprises a sequence of amino acids as set forth in SEQ ID NO:1.

7. The assay of claim 5, wherein said protein comprises a sequence of amino acids as set forth in SEQ ID NO:2.

8. The assay of claim 5, wherein said fluorophore is fluorescein isothiocyanate.

9. A diagnostic assay kit for detecting serum antibodies to a range of *Leptospira* serovars comprising:

a fluorescent antigen probe in an amount suitable for at least one assay and suitable packaging, said fluorescent antigen probe comprising a fluorophore conjugated to a purified and isolated protein comprising a sequence of amino acids that is at least 98.6% homologous with the sequence of amino acids set forth in SEQ ID NO:2.

10. The kit of claim 9, wherein said protein comprises a sequence of amino acids as set forth in SEQ ID NO:1.

11. The kit of claim 9, wherein said protein comprises a sequence of amino acids as set forth in SEQ ID NO:2.

12. The kit of claim 9, wherein said fluorophore is fluorescein isothiocyanate.

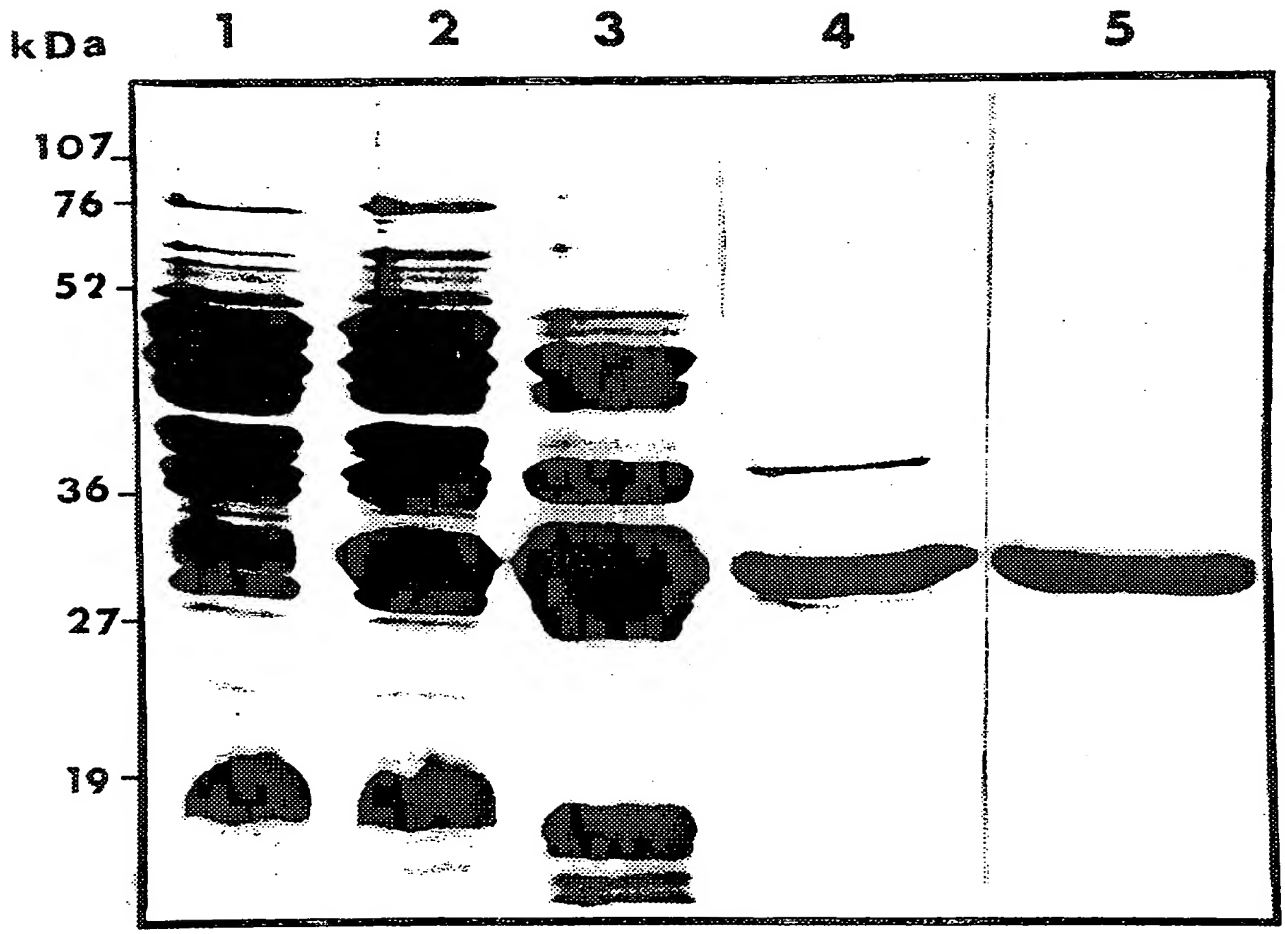

FIG. 1

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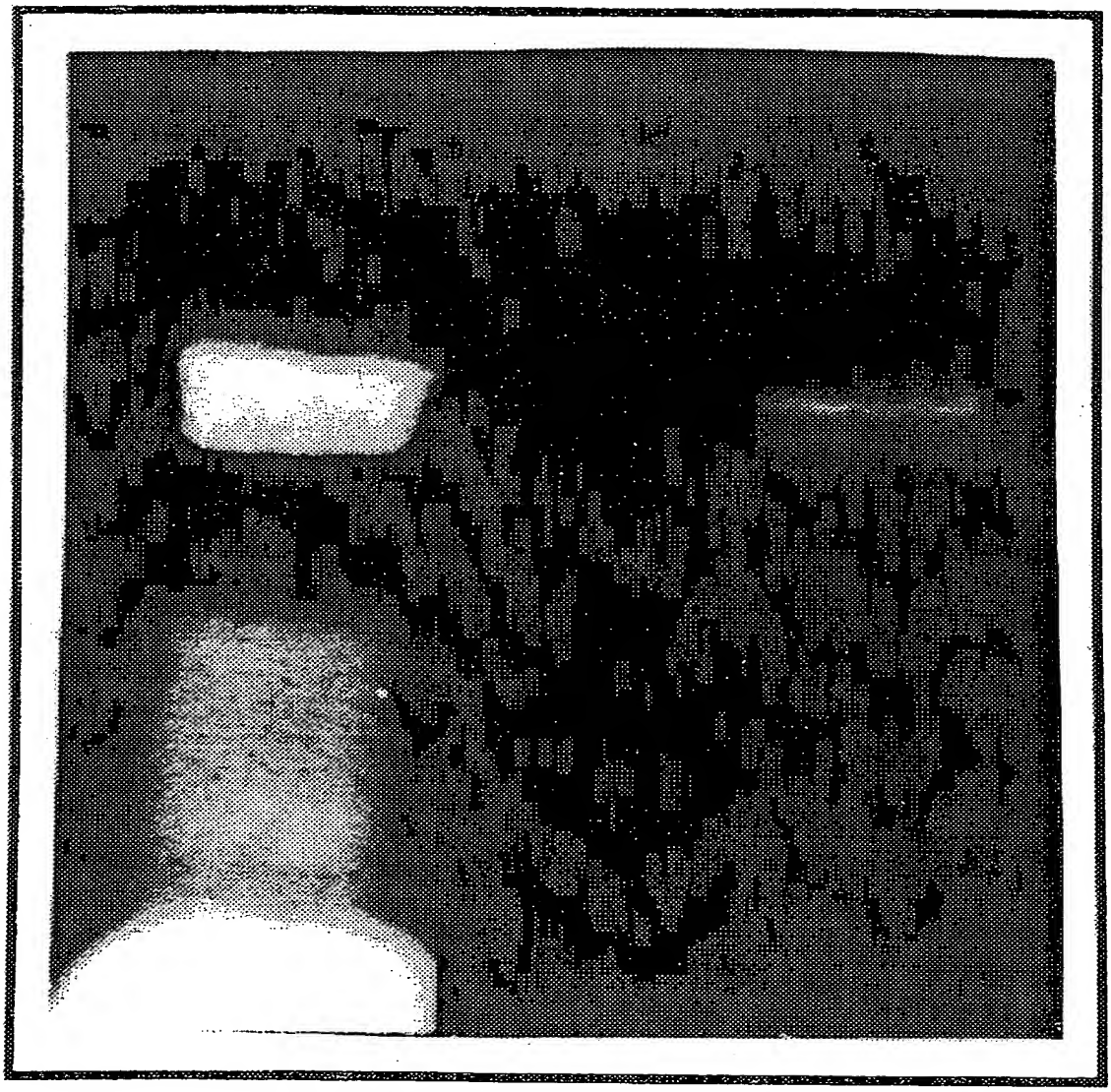

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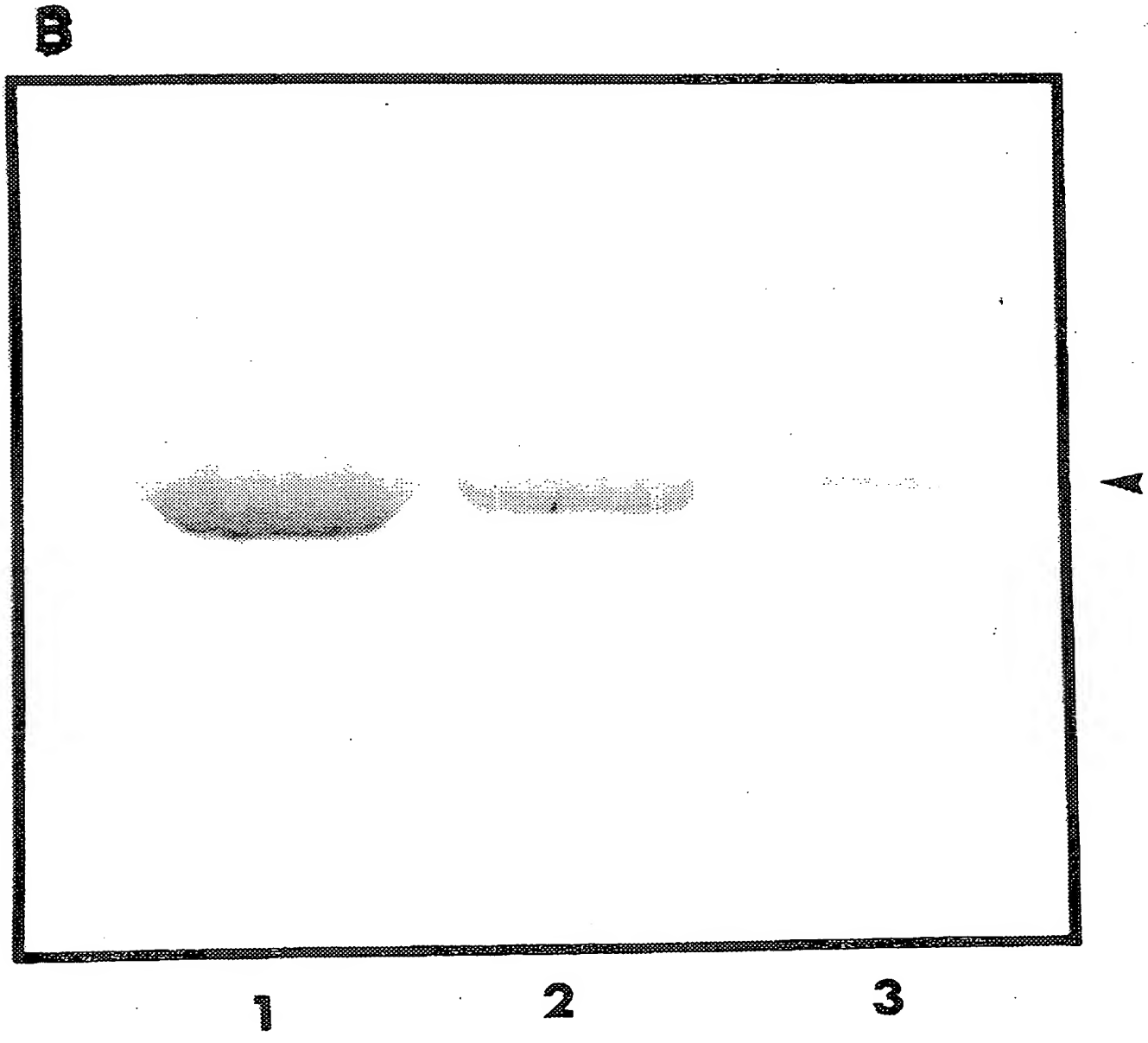
FIG. 2A



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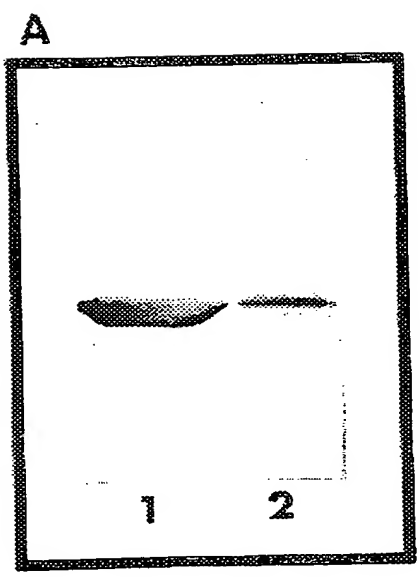

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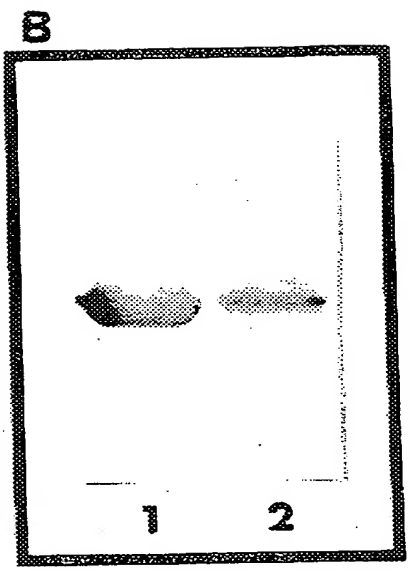

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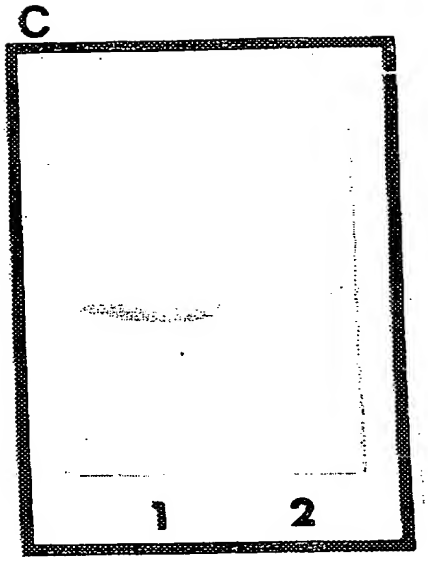

FIG. 3C

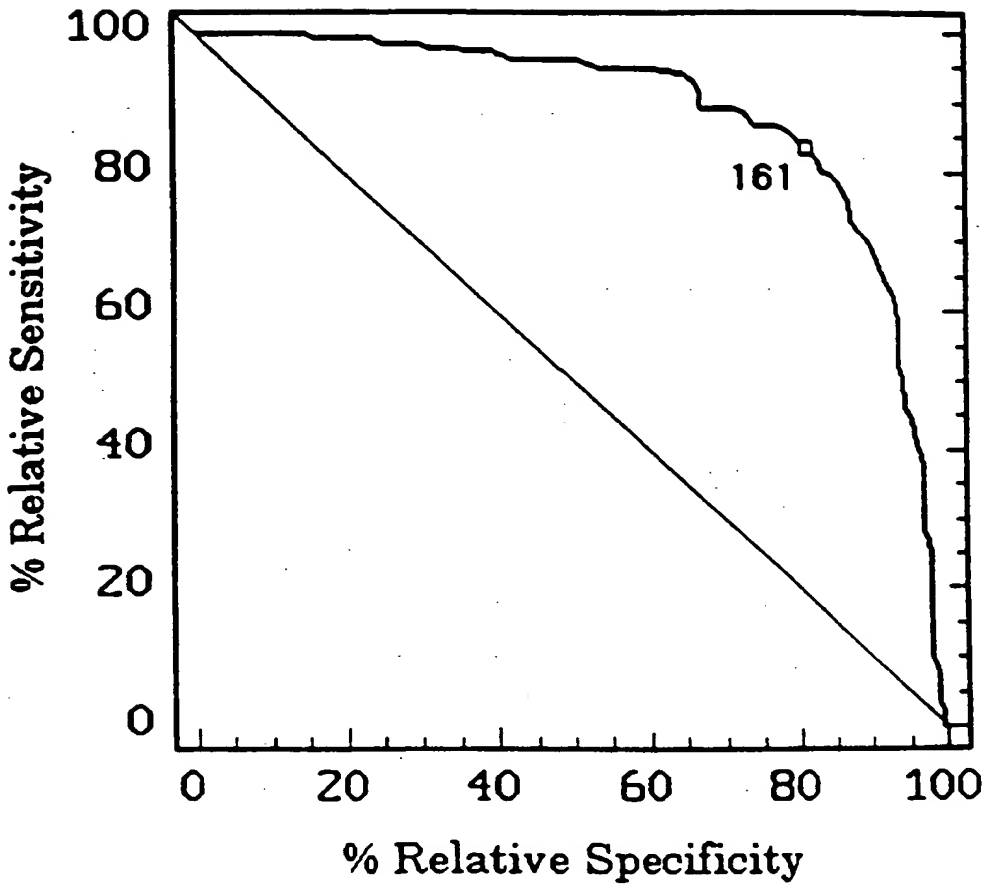
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Fig. 4

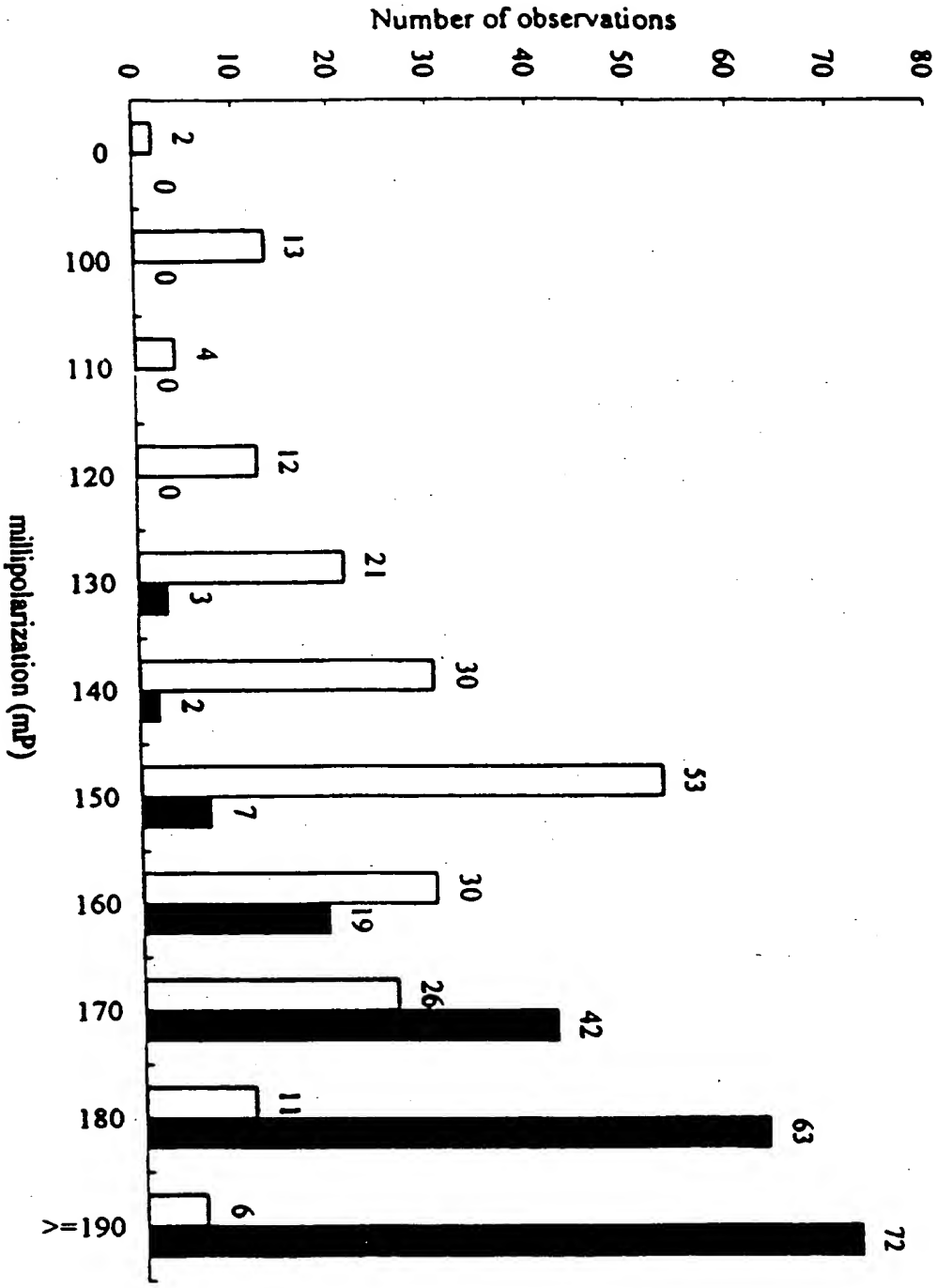
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FIG. 5

SEQUENCE LISTING

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<120> A Fluoresence Polarization-Based Diagnostic Assay for Leptospira Serovars

<130> Case No. 99,578

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22210

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G0IN 33/532, 33/531, 33/533, 33/53, 33/554, 21/76; C12Q 1/00
US CL :436/544, 543, 546,172,800; 435/4, 7.1, 7.2, 7.32
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/544, 543, 546,172,800; 435/4, 7.1, 7.2, 7.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, WEST, CAS, MEDLINE, BIOSIS
Search terms: flagellin, leptospira, antigen, probe, fluorescent label, fluorophore

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIN et al. Identification of a 35-kilodalton serovar-cross-reactive flagellar protein, FlaB, from *Leptospira interrogans* by N-terminal sequencing, gene cloning, and sequence analysis. Infection and Immunity. October 1997, Vol. 65, No. 10, pages 4355-4359, entire document.	1-12
Y	Database HCAPLUS, Accession No. 1995:34662, WOODWARD et al. 'Deoxynucleotide sequence conservation of the endoflagellin subunit protein gene, FlaB within the genus Leptospira', abstract, Vet. Microbiology. 1994, Vol. 40, Nos. 3-4, pages 239-251, entire abstract.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
21 DECEMBER 1999	24 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMA BASKAR Telephone No. (703) 308-0196